TRANSFECTION OF GENES ENCODING LYMPHOCYTE DIFFERENTIATION ANTIGENS:
APPLICATIONS IN VETERINARY IMMUNOLOGY

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ABSTRACT

The work conducted so far in this laboratory has demonstrated the application of the use of genes encoding lymphocyte differentiation molecules, in the isolation of homologous genes from other mammalian species, by the technique of cross-species DNA hybridization. The studies have also highlighted the use of transfection as a means of obtaining expression of genes, either from total genomic DNA or cloned in plasmids, which encode lymphocyte antigens. Preliminary work presented in this paper demonstrates the application of these technologies in the isolation and expression of genes for lymphocyte antigens from species in which the gene products have not been fully defined. We favour this approach because it may allow isolation and definition of important immunological molecules independently of the existence of specific antibodies. It therefore seems the most direct way to avoid the frustrating randomness in production of anti lymphocyte subset-specific monoclonal antibodies, and to shorten the time and effort needed to define the specificities of such reagents.

Furthermore, the cDNA clones isolated from alternate species (in this case the bovine) have a use in classical immunological studies apart from the application of antibodies made to their products in veterinary immunology. That is, comparisons of the DNA sequences of lymphocyte differentiation antigens from different species provide much important information about structural or functional elements of evolutionarily conserved proteins involved in generation of immune responses.

INTRODUCTION
The intensive research conducted on the immune system of Mus musculus, the domestic mouse, is of course justified in terms of developing a greater understanding of aspects relating to function and regulation of protective immune responses in all species. Parallel studies in humans, veterinary species and other laboratory animals continue to document the high degree of conservation of all functional levels of the mammalian immune system, ranging from organ structure and presence of discrete cell types, to functional molecules and ultimately the genes which encode them. Immunological research conducted on larger outbred animals, which do not lend themselves so readily to experimentation as do inbred strains of mice, relies heavily on continual cross reference to the more extensive elaboration of immune function and regulation described in laboratory animal models and humans.
One of the major limitations to the study of diseases of veterinary species is the lack of monoclonal antibodies which define the distinct lymphocyte populations and functional molecules that are now so readily identified in humans and mice. Major constraints in the production of such reagents are the randomness of their generation and the time and labour involved in defining their specificities once produced, usually by tissue distribution, target cell function or biochemical properties of the target molecules. Attempts to shortcut the production of specific typing reagents in larger animals by testing for a (cell type-specific) cross reaction with monoclonal antibodies to mouse or human lymphocyte subpopulations have often not been successful (W.C. Davis, personal communication; P. Lalor, unpublished data). Because it may be expected that homologous molecules to those defined by the antibodies in mice and humans do exist in these species, the lack of cross reactivity of the antibodies may be best explained by the presence of species-specific immunodominant determinants on these otherwise conserved structures.

Hence, rather than attempting to identify functionally homologous, immunological molecules across species by antibodies, it seems reasonable to adopt an alternative strategy that would allow identification of conserved molecules by methods based on recognition of invariant elements. One such strategy that we report here is the use of recombinant DNA technology to isolate and express from veterinary species, evolutionarily conserved gene homologues to those encoding immunological proteins of interest in humans and mice. The approach undertaken has been to use cloned genes encoding human or murine T lymphocyte differentiation molecules as probes for either the isolation or characterization of homologous genes in cattle. The studies can be envisaged at two distinct levels. First is the isolation from a DNA library of the bovine gene homologue which is subsequently transfected into and expressed in mouse L cells. The successful transfectants could be used to define the specificities of existing monoclonal antibodies or to make monoclonal antibodies specific for the expressed protein, if such reagents do not exist. Second, transfectants derived from genomic bovine DNA, which express the molecule recognized by monoclonal antibodies of undefined specificities, can be used as a source of mRNA to determine possible homology with cloned murine or human lymphocyte antigen genes.

Both approaches, one which would allow production of monoclonal antibodies of defined specificities and the second which would allow characterization of existing reagents, rely on the technologies of DNA transfection into eukaryotic cells and of cross-species DNA and RNA hybridization. This paper will discuss these technologies in the context of how they may be applied to the development of a more complete understanding of veterinary immunology.
APPROACH TO TRANSFECTION OF GENES ENCODING CELL SURFACE MOLECULES

Transfection is the process of DNA transfer into receptive cells. The process was first described in eukaryotic cells for the uptake and expression of naked poloma virus DNA by mouse embryo cells by Dimayorca et al (1969). Subsequent improvements in the methodology of transfection, including calcium phosphate precipitation of DNA (Graham and van der Eb, 1973) and co-transfection of DNA with a biochemically selectable marker (Wigler et al, 1978 and 1979) resulted in a method of gene transfer which has been readily applied to a wide range of eukaryotic genes. In this laboratory over the past few years, approximately 20 different human and murine cell surface molecules (Table 1) have been transfected into mouse L cells (Herzenberg et al., 1984; Hsu et al., 1984). Because of the details of the protocol used in this laboratory for transfection of genes have been published (Kavathas and Herzenberg, 1986) I shall limit the description of the method to those facets which, as we understand them, may be limiting in the application of transfection to veterinary immunology.

Within the recipient L cells, only a small fraction (approximately 0.1%) are receptive to the uptake, integration and expression of the donor DNA. Hence in establishing transfectants from genomic DNA, in which again approximately 0.1% of the transfectants express the gene of interest (Perucho et al., 1980; Robins et al., 1981), the absolute frequency of expression of the desired transfected gene in the L cells is in the order of 10-6. We therefore use a co-transfection system, modeled after that first described by Wigler et al. (1979) in which mouse L cells which lack the thymidine kinase (TK) gene (LTK-) are transfected

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**TABLE 1**

Lymphocyte surface molecules transfected into mouse L cells

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>T Cell Molecules</th>
<th>B Cell Molecules</th>
<th>OTHER Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Leu-1/C05</td>
<td>BLA-1</td>
<td>Class I MHC</td>
</tr>
<tr>
<td></td>
<td>Leu-2/C08</td>
<td>BLA-2</td>
<td>NGF Receptor</td>
</tr>
<tr>
<td></td>
<td>Leu-3/C04</td>
<td>Thy8</td>
<td>Transferrin Receptor</td>
</tr>
<tr>
<td>Mouse</td>
<td>Ly-1</td>
<td>BLA-1</td>
<td>Trop-1 and 2</td>
</tr>
<tr>
<td></td>
<td>Ly-2</td>
<td>BLA-2</td>
<td>Class I MHC</td>
</tr>
<tr>
<td></td>
<td>L3T4</td>
<td>Thy8</td>
<td>Fc Receptor</td>
</tr>
<tr>
<td></td>
<td>Thy-1</td>
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</tr>
<tr>
<td></td>
<td>T200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with both genomic DNA and the purified chicken TK gene. Transfectants are grown in selective HAT medium. Because thymidylicate nucleotide synthesis is blocked in cells by aminopterin, only the minor population of cells permissive for transfection (which have accordingly taken up both the TK gene and approximately 1000 kb of genomic DNA) (Peruch et al., 1980; Robins et al., 1981) will be capable of utilizing the exogenous thymidine via the thymidine kinase enzyme. Transfected cells are accordingly rescued from aminopterin poisoning by overcoming the block in purine metabolism. By the simple positive selection for TK^+ transfectants, the frequency of cells expressing transfected genes is increased a thousand fold.

The transfectants expressing specific cell surface molecules can be isolated by staining with monoclonal antibodies and selection by the fluorescence-activated cell sorter (FACS). The specific transfectants are usually clonally selected after 2 cycles of staining and sorting. The use of the FACS for selection reliably permits recovery of specific transfectants at frequencies as low as 10^-6 within the total population of transfectants. It further allows, by electronic subtraction of cell autofluorescence, detection and selection of specific transfectants which express only very low levels of surface protein (Alberti et al., 1986). And interestingly, it also permits the isolation of specific transfectants which express higher levels of surface protein. Studies in this laboratory have shown that such high expressors often result from spontaneous amplification of the transfected gene within the recipient cell (Kavathas and Herzenberg, 1983).

The frequency of specific co-transfectants resulting from use of cloned genes (for cell surface proteins) as a source of donor DNA, together with TK, is of course much higher than the 10^-3 obtained for genomic DNA. Usually between 30% to 80% of the HAT selected TK^+ transfectants stably express the gene product (S. Alberti, unpublished data). The importance in the application of this finding in our studies with bovine genes is discussed below.

EXPRESSION BY L CELLS OF TRANSFECTED LYMPHOCYTE DIFFERENTIATION ANTIGENS

The major consideration in attempting to establish transfectants for any given lymphocyte surface protein relate to the source of DNA used, the nature of the gene itself, and in some instances, the functional or developmental nature of the recipient cell. Because all transfectants I will describe were prepared in mouse fibroblastic L cells, I will consider the latter point only briefly. It has been shown that certain genes, for example the immunoglobulin genes (Stafford and Queen, 1983; Morrison and Oi, 1984), require the presence of tissue specific enhancers for functional expression. Attempts to transfet and express rearranged 1g genes into L cells have generally failed, whereas transfer of the same genes into B cell lines, which presumably produce the trans-acting
regulatory enhancer proteins, results in expression of the gene. However, for the vast majority of lymphocyte proteins transfected to date, there has been no evidence for the requirement of such tissue-specific enhancer proteins.

Whether protein is produced and transported to the surface of the transfecants as a functional protein is due in part to the number of distinct genes encoding polypeptide chains involved in membrane protein structures. If the cell surface expression of a molecule is dependent on proteins encoded by two or more unlinked genes, then the expected frequency of co-transfections which would take up and express both genes would be in the order of $10^{-9}$ to $10^{-10}$ of the original, unselected transfecants. Such frequencies are below the levels of meaningful detection in eukaryotic transfection systems. However, the high frequencies of transfection obtained using cloned genes (see above) allows for simultaneous, multiple transfections, and resultant reconstruction of heterodimeric proteins in L cells. In such ways, functional class II MHC molecules composed of two distinct polypeptide chains, and both an immunoglobulin molecule and a T cell receptor, each with antigen specificity encoded by 2 distinct genes, have been successfully transfected into recipient cells (Morrison and Oi, 1984; Dembic et al., 1986; Germain and Malissen, 1986). As described above, the recipient cell for Ig transfection should be of a B cell lineage because of the need for lineage specific trans-acting factors.

Interestingly, class I MHC molecules, also heterodimers, and murine Lyt-2 (which is expressed on T cells as a heterodimer in association with the Lyt-3 molecules), are readily expressed on L cells transfected with single genes. The presence in L cells of constitutively low levels of beta-microglobulin (which associates with the polymorphic class I heavy chain) accounts for the surface expression of both mouse and human class I molecules (Woodward et al., 1992). Transfected Lyt-2 protein chains, in the absence of Lyt-3, associate as homodimers prior to expression on the cell surface (Tagawa et al., 1986).

The size of a gene may also be a limitation in its transfectability. The human Leu-3/CD4 molecule, which in itself is not an inordinately large molecule (being Mt 59kd), is encoded by a gene which is approximately 33kb long, with some 20kb between the first and second exons (D. Littman, personal communication, 1986). As a consequence, the frequency of transfecants which express a complete cell surface Leu-3/CD4 molecule is far lower than frequencies for specific transfecants of smaller genes (approximately 5kd) encoding molecules such as Leu-2/CD8 or Lyt-2 and Ly-1 (S. Alberti, C. Hsu and P. Kavathas, unpublished data).

And a final consideration in primary transfection, and expression of uncharacterized genes, is the source of DNA and associated status of the specific gene. For those such as immunoglobulin genes and T cell receptor genes where a genomic rearrangement is necessary to produce a functional gene,
transfection with a source of unarranged (germ-line) DNA will certainly transfer the gene, but will not result in expression of functional protein. Other sources of DNA, for example from trophoblasts (the cells which form the immunological barrier between the placenta and the developing foetus) or from choriocarcinoma cell lines derived from them, where gene expression for most molecules may be permanently shut down by mechanisms such as DNA methylation, may also be incapable of transferring genes which can produce polypeptide chains (Herzengerg et al., 1984; and Alberti and Herzenberg, manuscript in preparation). Surprisingly, however, it does appear that DNA isolated from almost any tissue source, whether of lymphoid origin or not, is highly efficient at transferring expressible genes encoding most, if not all, lymphocyte differentiation antigens, not limited by the other restrictions described above. Perhaps the best example of this is DNA prepared from (recipient) fibroblastic L cells, which do not express T or B lymphocyte differentiation antigens, will transf ect readily expressible genes for these molecules into the same L cell line (Hsu et al., 1984). Hence transfection of DNA gives rise to expression, in the recipient cells, of genes which in the donor cells were regulated for non-expression.

The same general restrictions apply to transfection of L cells with cloned genomic genes (in plasmids or phage) as for whole genomic DNA, although passage of the gene through bacteria may alter methylation patterns. However, different gene regulatory restrictions apply in transfections with cDNA gene clones, which by virtue of being DNA copied from an mRNA template, lack upstream and possibly downstream regulatory sequences (such as 5’ promoter site, including enhancer sequences, initiation site and ribosomal binding site, and 3’ polyadenylation sites), and intron/exon gene structure contained in genomic sequences. To circumvent the loss of the regulatory sequences, cDNA gene libraries are ideally prepared in an expression vector, such as pcDV, which provides the promoter site and polyadenylation site missing in the cDNA (Okayama and Berg, 1982). Alternately, isolated cDNA clones prepared in non-expression vectors such as lambda GT10 can be subcloned into expression vectors prior to transfection.

The loss of intron/exon structure in cDNA clones can limit the heterogeneity of polypeptide chain formation upon transfection, for genes where differential splicing of the genomic DNA (regulated by intron sequences) may give rise to different forms of the polypeptide. A classic example of this exists for the murine Lyt-2 gene. The genomic DNA (containing a single Lyt-2 gene), upon transfection, gives rise to two distinct forms of the polypeptide (Mr 38kd and 34kd) within the same cell. The two forms are translated from two distinct mRNA species differing by 31 bases, which arise due to differential splicing of the nuclear mRNA. Individual cDNA clones have been isolated, which depending on the mRNA species which acted as the original template, can transfect either the 38kd
or the 34kd form of the molecule, but not both (Tagawa et al., 1986; Zamoyska et al., 1986).

The fact that both genomic-derived forms of the Lyt-2 molecule are seen in transfected L cells, and that both forms show similar glycosylation patterns to the two forms of the molecule seen in mouse thymocytes, indicates that the post-translational processing of the molecule in L cells is not distinguished from that in T cells (Nakauchi et al., 1985; Tagawa et al., 1986). This appears to be generally true, in that transfected glycoproteins have shown when tested similar molecular weights to the native molecules expressed in lymphocytes. Furthermore, for several cell surface molecules tested, the transfected molecules maintained identical functions, specificities, specific recognition sites or ligand binding capacity, as the native proteins. Examples are: (1) the preservation by transfected murine and human class I MHC molecules of the recognition site for alloreactive CTL clones (Germain and Malissen, 1986); (2) the acquisition of antigen presenting cell function of class II MHC transfectants (Germain and Malissen, 1986); (3) the maintenance of IL-2 binding capacity by the transfected IL-2 receptor (Kond et al., 1986).

In summary, it seems that transfection of lymphocyte differentiation antigens into mouse L cells is a remarkably generalized phenomenon, with surprisingly few restrictions limiting the functional expression of most molecules assessed to date. The L cells themselves are capable of processing the introduced polypeptide chains to produce functional, mature proteins, in a manner analogous, if not identical, to processing in lymphocytes.

**ISOLATION AND TRANSFECTION OF GENE HOMOLOGUES**

The products of the major histocompatibility locus were first identified serologically in humans by use of alloantisera, and shown to be of central importance in the rejection of tissue transplants. The MHC-encoded lymphocyte surface molecules were subsequently identified as 2 classes of glycoproteins, expressed mostly on cells within the lymphoid system and generally absent (class II) or at lower levels (class I) on most somatic tissues of the body. Alloantisera produced in mice and other species recognized cell surface glycoproteins with a similar tissue distribution and function in transplantation rejection to those observed in humans. The close similarity in structure and function of these molecules suggested the widespread presence in vertebrates of a set of highly conserved genes. The existence of gene homologues, in all species tested, for class I and class II molecules has now been extensively documented by cross species DNA hybridization, gene cloning and DNA sequencing (Danska and McDevitt, 1986). In fact, the presence of homologous genes, encoding what may be functionally analogous proteins to mammalian class II MHC molecules, has been described even in higher order invertebrates, such as...
tunicates (Danska and McDevitt, 1986). Similarly, genes encoding immunoglobulins have been shown by the methods of DNA hybridization and sequencing, to be conserved in many vertebrates (Danska and McDevitt, 1986; Litman et al., 1982; Litman et al., 1983).

The general applicability of these findings for other immunological molecules with structural and functional properties highly conserved between species (for a more complete listing, see Williams and Barclay, 1986), is now beginning to be exploited. Homologies have been proposed for several sets of murine and human lymphocyte molecules, many of which are associated with restricted functions of T cell subsets. The human lymphocyte antigens Leu-1/CD4, Leu-2/CD8, and Leu-3/CD4 have biochemical structure and restricted cellular expression analogous to the murine Ly-1, Lyt-2 and L3T4 molecules, respectively (Ledbetter et al., 1981; Dialynas et al., 1983). The human Leu-4/T3 molecule, associated with the T cell receptor, has a proposed homology to an as yet unnamed molecule associated with the murine T cell receptor (Allison and Lanier, 1985).

The homology at the DNA level of these molecules in different species has now begun to be characterized. Research conducted in this laboratory and in collaboration with Nancy Jones and Jack Strominger at the Dana-Farber Cancer Institute has shown that significant homology exists between the isolated Leu-1/CD5 and Ly-1 genes (Huang et al., 1986; Jones et al., 1986) and the Leu-2/CD8 and Lyt-2 genes (Nakauchi et al., 1985). The murine Ly-1 and Lyt-2 genes were both in fact isolated by use of the homologous human gene probes. The homology between Leu-2/CD8 and Lyt-2 has also been independently reported (Zamoyska et al., 1985).

The Lyt-2 gene was isolated from a mouse thymocyte cDNA library using 1.7Kb Leu-2/CD8 cDNA probe (itself cloned by transfection and cDNA subtraction (Kavithas et al., 1984; Litman et al., 1985) under conditions of low stringency hybridization. The isolated murine gene was confirmed to encode Lyt-2 by use of the transfection systems described above. First, the isolated mouse gene strongly hybridized with mRNA from Lyt-2 transfecents (established by selection of specific genomic DNA transfecents using anti Lyt-2 antibody and the FACS). And second, the cDNA clones, when transfected into L cells, expressed the murine Lyt-2 molecule, as identified by monoclonal anti Lyt-2 immunoprecipitation and two-dimensional gel analysis (Nakauchi et al., 1985). From sequence data obtained for the two cDNA clones, the overall homology between the two coding regions is 56%. When the domain-like structure of the two molecules is considered, homology is more conserved for segments of the gene which encode the intracytoplasmic, transmembrane and first external portions of the molecule. Overall homology for these segments is 64%. The major external domain is less conserved (DNA homology 42%), and may reflect the co-evolution of this segment.
of the molecule with the MHC class I molecules (Zamowska et al., 1985; Nakauchi et al., 1985).

Similarly, the murine Ly-1 gene was isolated from a mouse cDNA library by use of a 1.0 kb cDNA human Leu-1/CDS probe, itself originally isolated by Nancy Jones and Jack Strominger by construction of an oligonucleotide probe based on the N-terminal amino acid sequence of Leu-1/CDS (Jones et al., 1986; Huang et al., 1986). The overall DNA homology for the coding sequence of the two genes is 73%.

We now report the isolation of the bovine Leu-2/CDB gene homologue from a thymic cDNA library prepared in the Okayama-Berg expression vector, pCDV. The library was a kind gift of Dr. Ken-ichi Aria at DNAx, Palo Alto. The library was originally screened with the 1.7 kb Leu-2/CDB cDNA probe used to isolate the murine gene, under identical low stringency hybridization conditions (Nakauchi et al., 1985). From 5 x 10^4 colonies screened, we isolated 18 clones which showed strong hybridization with the probe. DNA from twelve of these clones, when purified, digested with various restriction enzymes and electrophoresed on agarose gels, showed a similar restriction fragment pattern, with only one fragment varying in size between clones. We expect that the variable band represents the 5' RE fragment in each clone, and its variable length reflects the heterogeneity of size of the cDNA species produced in the original construction of the library. The frequency of representation of clones of bovine Leu-2/CDB detected in the library (representing the frequency of transcripts in the thymus) was therefore approximately 0.04%.

However, approximately 5 x 10^5 colonies from the library had to be screened before isolation of a clone which contained the full sequence for the mature peptide. The screening was conducted in two further successive colony hybridizations, each using as a probe, a 5' fragment from the previously isolated bovine Leu-2 cDNA clone. The bovine Leu-2 cDNA clone (M2-c15) is 2.1kb in length. By comparison of the sequence of the 5' end of the clone with the sequences of both Lyt-2 and Leu-2/CDB we conclude that the clone includes all the sequence necessary for the mature peptide. However, it appears that the clone lacks an estimated 6 base pairs for the N-terminal end of the leader peptide, including the obligatory ATG codon. The leader peptide is a small hydrophobic moiety contiguous with the mature peptide which ensures successful transport of the protein through the ER apparatus of the cell. The leader peptide, which is 27 amino acids long in Lyt-2 and 19 residues in Leu-2/CDB, is then cleaved from the mature protein, before cell surface expression.

Therefore, before attempting to transfect the M2-c15 clone into L cells, we will construct an oligonucleotide which, when ligated to the 5' end of the clone, will generate the two expected N-terminal leader amino acids including, of course, the f-methionine encoded by the ATG. The construct will be co-
transfected into LTK- cells with the chicken TK gene, using conditions established in this laboratory which yield approximately 50% of the HAT-selected transfectants expressing the specific gene product. The transfectants will be screened for the presence of bovine Leu-2 specific mRNA, and mRNA-expressing transfectants will be used to immunize both BALB/c and C3H/He mice (from which the recipient L cell line was originally isolated). Sera from these mice will be tested for the presence of antibodies which react with bovine T cells, and spleen cells from positive mice will be fused to the myeloma partner, SP2/0, for generation of monoclonal antibodies.

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